IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: HADLACZKY et al.

Serial No.: 09/096,648 Group Art Unit: 1632

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For: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES

DECLARATION PURSUANT TO 37 C.F.R. §1.132

The Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Carl Perez declare as follows:

1) I am currently Director of Projects at Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A IW9. I have held this position since March 10, 1997. I earned a doctoral degree in biophysics at the University of California at Berkeley in August 1984.

2) In my position at Chromos Molecular Systems, Inc., (hereinafter Chromos) I have been extensively involved in projects designed to generate transgenic animals using satellite artificial chromosomes. Using methods and materials described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have transferred satellite DNA-based artificial chromosomes containing multiple copies of the *lacZ* and *hph* (hygromycin phosphotransferase) genes into the pronucleus of fertilized bovine oocytes to produce transgenic embryos. Fluorescence *in situ* hybridization (FISH) analysis of blastocysts using probes specific for the artificial chromosome revealed that 27% of the embryos obtained after pronuclear injection were transgenic and that, on average, 27% of the cells of the embryos contained the artificial chromosomes as discrete chromosomes.

IDM Considered 2/23/01

The results of these analyses demonstrate that satellite DNA-based artificial chromosomes as described in the above-referenced application can be used in standard methods employed in the generation of transgenic animals to yield viable transgenic bovine embryos. The embryos contain within their cells

intact, heterologous gene-containing artificial chromosomes as autonomous, stably replicating, extrachromosomal elements.

A description of the above-referenced methods, embryos and results follows.

I. Materials and methods

A. Satellite DNA-Based Artificial Chromosomes

Satellite DNA-based artificial chromosomes were obtained from a mouse-hamster-human cell line containing a 50-60 Mb micro-megachromosome carrying the anti-HIV *gag* ribozyme and the hygromycin phosphotransferase and β-galactosidase genes. The cell line was generated in accordance with methods described in detail in the above-referenced application.

Specifically, the H1xHe41 cell line (mouse-hamster-human hybrid cell line carrying a megachromosome and a single human chromosome with CD4 and neo^r genes), which is described in the above-captioned application on page 68, lines 9-15, was subjected to repeated BrdU treatment followed by single cell cloning to yield the mM2C1 cell line. As described in the above-referenced application, the H1xHe41 cells are ultimately derived from EC3/7C5 cells that had been co-transfected with pCH110 and pH132. These plasmids carry the β-galactosidase-encoding gene (*lacZ*), which is linked with the SV40 promoter, (pCH110) and the hygromycin-resistance gene (*hph*) and anti-HIV *gag* ribozyme under control of the β-actin promoter (pH132; see pages 53-54 of the above-captioned application for a description of construction of pH132).

The mM2C1 cell line contains the ~60 Mb megachromosome containing the anti-HIV *gag* ribozyme and the *hph* and *lacZ* genes. mM2C1 cells were fused with chinese hamster ovary (CHO) cells by microcell fusion to generated CHO-E4-20 cells as follows. Mitotic cells were harvested from colchicine-treated mM2C1 cells and centrifuged through a percoll gradient in the presence of cytochalasin-B. Microcells were passed through successive filters and overlaid for 20 minutes onto recipient CHO cells and treated with polyethylene glycol. Selection for clones containing satellite DNA-based artificial

chromosomes was based on expression of β-galactosidase using hygromycin resistant growth techniques. Single-cell cloning by limiting dilution was repeated several times to yield the CHO-E4-20 cell line that contains two intact, functional ~60 Mb megachromosomes. The cell line was grown under standard conditions in MEM-α medium under selective (0.15 μg/ml hygromycin) conditions.

B. Isolation and Purification of Satellite DNA-Based Artificial Chromosomes

The satellite DNA-based artificial chromosomes were purified from CHO-E4-20 cells using flow cytometry generally as described in the above-referenced application. The artificial chromosomes were first isolated from the cells as follows. Cells were plated in 150 mm tissue culture dishes and supplemented with fetal calf serum and hygromycin B. After 24 hours, exponentially growing cells were blocked in mitosis with colchicine (1.0 μg/ml) for 7 hours before harvest. Mitotic cells collected by washing were swollen in a hypotonic buffer of 75 mM KCl for 10 min at room temperature. After swelling, the cells were transferred to the polyamine buffer (80 mM KCl, 70 mM NaCl, 0.1% β-mercaptoethanol, 15 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 0.2 M spermine, 0.5 M spermidine, and 0.25% Triton X-100, adjusted to pH 7.2) and incubated on ice. Shearing of the cell membranes was achieved by gently drawing the cell suspension up and down a 22-gauge needle attached to a 10-ml syringe. Hexylene glycol (2%)/200mM glycine buffer was added to an equal volume of the polyamine buffer containing the released chromosomes giving a final volume of 20 ml. Prior to staining, the chromosome preparation was centrifuged at 100g for 1 minute to remove cellular debris.

The chromosome suspension (supernatant) was removed to a fresh tube and stained with Hoechst 33258 (2.5 μg/ml), chromomycin A3 (50 μg/ml) in the presence of 2.5 mM $MgCl_2$. Samples were stored at 4°C for a minimum of 2 hours. Fifteen minutes before flow cytometric sorting, 10 mM sodium citrate and 25 mM sodium sulfite were added. A final concentration of 15-20 million

chromosomes per ml was achieved. All chromosome preparations were filtered through a 35-μm nylon mesh and stored on ice until sorted.

Purification of the satellite DNA-based artificial chromosomes from the chromosome preparation was performed on a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with a Turbo-Sort Option and two Innova 306 lasers (Choerent, Palo Alto, CA). Hoechst 33258 was excited with the primary UV laser beam and excitation detected in FL1 using a 420-nm band-pass filter, whereas chromomycin A3 was excited by the second laser set at 458 nm, and fluorescence detected in FL 4 by using a 475 nm long-pass filter. The sheath buffer used in the sorting procedure contained 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 100 mM NaCl, 30 μM spermine and 70 μM spermidine. Flow-sorted satellite DNA-based artificial chromosomes were pelleted by centrifugation of a 1-ml sample containing $\sim 10^6$ chromosomes at 2500 x g for 15 minutes at 4°C, and finally retaining only 10-20 μl of loose pellet.

C. Preparation of *In Vitro*-Fertilized Bovine Oocytes

Immature bovine oocytes were harvested as described by Keefer *et al.* [(1994) *Biol. Reprod. 50*:935-939]. Bull sperm was prepared for *in vitro* fertilization (IVF) as described by Goto *et al.* [(1990) *Veterinary Record 127*:517-520]. The oocytes were fertilized *in vitro* as described by Navara *et al.* [(1994) *Develop. Biol. 162*:29-40].

D. Microinjection of Satellite DNA-Based Artificial Chromosomes

For bovine pronuclear injections, non-filamented borosilicate glass micropipettes were pulled to an inner diameter of 1.7 - 2.4 μm and a corresponding outer diameter of 2.3-3.2 μm and bevelled to a 30° angle (Humagen, Charlottesville, Virginia). Oocytes were deposited in a 12 μl volume of injection medium on a depression microscope slide. The injection medium consisted of one part concentrated satellite DNA-based artificial chromosomes in sheath buffer and 3 parts M2 medium (Sigma), and was covered by mineral oil. Pronuclei and satellite DNA-based artificial chromosomes (approximately 2 μm x

1 μm) were visualized using Leica differential interference contrast optics. One or more artificial chromosomes were frontloaded into the tip of the needle by applying suction on the microinjection needle using a manual air-driven SAS11/2-E equilibrating syringe (Research Instruments, Cornwall, England), and injected into the male pronucleus.

E. Fluorescence *in situ* Hybridization

Single bovine embryos that were generated by injection of satellite DNA-based artificial chromosomes into fertilized bovine oocytes were analyzed by whole-mount fluorescence *in situ* hybridization (FISH). Bovine embryos were cultured and arrested with colcemid as described by Garside *et al.* [(1985) *Experientia 41*:1183-1184]. The arrested embryos were processed for FISH as described by Harper *et al.* [(1994) *Hum. Reprod. 9*:721-724].

II. Analysis of Injected Bovine Embryos

A. Survival

Approximately 16% of bovine embryos injected with buffer alone developed to blastocysts seven days after pronuclear injection. Approximately 20% of bovine embryos developed to blastocysts seven days after pronuclear injection of the satellite DNA-based artificial chromosomes. Therefore, it was determined that the artificial chromosome did not affect early embryo development.

B. Presence of Satellite DNA-Based Artificial Chromosomes

To look for the presence, and to evaluate the fate of injected satellite DNA-based artificial chromosomes, FISH was performed on whole embryos that had been produced by injection of oocytes with artificial chromosomes. Bovine blastocysts were probed with mouse major satellite DNA. This murine nucleic acid sequence does not cross hybridize with bovine genomic DNA. The satellite artificial chromosome was present in 27% of bovine embryos analyzed. Embryos exhibited varying degrees of mosaicism for the presence of satellite DNA-based artificial chromosomes, which were detected in 12% to 50% of cells scored for each positive embryo. On average, 27% of the cells scored for

each positive embryo contained a satellite DNA-based artificial chromosome. Although the majority of nuclei contained in a whole-mount embryo are interphase nuclei, there is usually (i.e., in approximately 3-10% of the blastomeres scored) at least one metaphase cell detectable in the embryo. In analysis of metaphase chromosomes detected in the embryos, no translocation events between the artificial chromosome marker sequences and native chromosomes were observed, and the artificial chromosomes appeared to be intact at the level of FISH analysis.

III. Summary and Conclusions

Using satellite DNA-based artificial chromosomes and methods described in the above-referenced application, as well as standard methods of oocyte and embryo manipulation known in the art and described herein, it has been possible to generate transgenic bovine embryos that stably maintain satellite DNA-based artificial chromosomes as discrete elements that remain separate from the host genome. The artificial chromosomes were able to undergo mitotic segregation in early embryonic development in the absence of selective pressure, and did not show evidence of integration into the host genome.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Carl F. Perez

Carl Perez

Date: Jan. 3, 2001